United States Environmental Protection Agency

LABORATORY ANIMAL PROJECT REVIEW

Please note:

1. All information in this LAPR is considered privileged and confidential by the IACUC and regulatory authorities.
2. Approved LAPRs are subject to release to the public under the Freedom of Information Act (FOIA). Do not include proprietary or classified information in the LAPR.
3. An approved LAPR is valid for three years.

LAPR Information

LAPR Title:	The contribution of neuroendocrine stress response in pulmonary and systemic health effects of air pollutants in rats
LAPR Number:	19-02-002
Principal Investigator	Exemption 6
Author of this Document:	Exemption 6 /RTP/USEPA/US
Date Originated:	12/19/2015
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Date Approved:	02/22/2016
Date Closed:	

APPROVALS

APPROVER	NAME	APPROVAL DATE	COMMENTS	
	Exemption 6 TP/USEPA/US by Exemption 6 /RTP/USEPA/US	02/22/2016	DMR reviewed.	
	Exemption 6 RTP/USEPA/US by Exemption 6 /RTP/USEPA/US	02/22/2016	DMR	

Administrative Information

1. Project Title (no abbreviations, include species):
The contribution of neuroendocrine stress response in pulmonary and systemic health effects of air pollutants in rats

Is this a continuing study with a previously approved LAPR?
Yes
Please provide the previous LAPR# 16-03-003 and 18-01-001

2. Programatic Information
a. What Program does this LAPR support? Please provide the Research Program, Project, Task Number and Title.
ACE PEP1.2; Health Impacts of Regional Complex Air Pollution Mixtures in At-Risk Populations
ACE PEP2.1; Understanding Modifiable Factors that Influence Air Pollution Related Public Health Impacts in Healthy and at Risk Populations to Inform Development of Mitigation Strategies at the Individual and Community Level
b. What is the Quality Assurance Project Plan (QAPP) covering this project?
IRP-NHEERL-RTP/EPHD/CIB/2015-001(Autophagy)-r1
IRP-NHEERL-RTP/EPHD/CIB/2016-001-r1

3. EPA Principal Investigator/Responsible Employee:

Principal Investigator	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6 Exemption 6/RTP/USEPA/US	EPHD ***Branch*** CIB	MD

4. Alternate Contact:

Alternate Contact	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	Exemption 6 ***Lotus Notes Address*** Exemption 6 Exemption 6 Exemption 6 Exemption 6/RTP/USE	EPHD ***Branch*** CIB	MD

SECTION A - Description of Project

1. Explain the study objective(s) in non-technical language such that it is understandable by non-scientific persons. Explain how the benefits from the knowledge gained from this research outweigh the costs to the animals used in this research. If this is a continuing study from a previous LAPR, briefly justify the continuation. Please spell out all acronyms and abbreviations with their initial use.

Stress in day-to-day life plays a central role in a variety of health issues such as cardiovascular, mental, reproductive and metabolic diseases. The stress response in body is induced by fear, psychological factors, and also by sudden injury, infection or burn. When the body experiences stress, the brain orchestrates a series of events through activation of sympathetic nervous system and hormonal stress axis called hypothalamus-pituitary-adrenal-axis (HPA-axis), which increases the release of two primary stress hormones from adrenal glands, epinephrine and corticosterone (cortisol in humans). Epinephrine is made in adrenal medulla and corticosterone is made in adrenal cortex. These hormones regulate two survival mechanisms, such as an immune response (to fight invading pathogens/foreign substances) and metabolism to channel energy at the site of stress in the body. Virtually all organs in the body are engaged in mediation of this stress response. This is accomplished by the receptors for these hormones in all organs in the body to allow for these hormones to exert their action. Overactivity of this system or its malfunction can lead to adverse health effects.

Under LAPR 16-03-003, we have recently shown for the first time that exposure to air pollutant, ozone can induce this classical hormonal stress response in rats and compared this to human data. We have also shown that when adrenal glands are surgically removed from rats, the systemic and lung ozone effects are diminished suggesting critical role of stress hormones in mediating all ozone effects. This observation challenges the currently established belief that ozone induced lung injury is mediated solely by the oxidative action of ozone on lung surface. The goal of this project is to examine how pulmonary and systemic metabolic as well as immune effects of ozone are modulated by circulating stress hormones and their receptors in rats using pharmacological inhibitors and activators of stress hormone receptors.

Specifically, we will examine the role of beta adrenergic receptors (BAR; for epinephrine) and glucocorticoid receptors (GR; for corticosterone) using receptor specific antagonists and agonists. We have shown in our earlier studies that ozone induces systemic metabolic impairment and lung injury/inflammation through endocrine stress response activation and release of epinephrine and corticosterone from adrenals. Epinephrine modulates its effects by binding to BAR (abundant in the lung and other organs) while corticosterone induces cellular effects by binding to GR. The inhibition and activation of these receptors through selective and non-selective antagonists are widely used in the biomedical field to counter adverse metabolic and inflammatory conditions. Understanding how these receptors modulate ozone-induced metabolic impairment and lung/injury inflammation is highly critical in supporting risk assessment of not only ozone but also other pollutants. This study also may provide causal evidence for a common mechanistic pathway involving a hormonal stress response.

We will conduct three experiments which do not involve surgery.

1) In the first experiment, we will use two antagonists widely used to antagonize the activity of GR and BAR and thus, the effects of stress hormones in modulating ozone-induced systemic and pulmonary effects. These are propranolol for BAR and Mifepristone for GR antagonism. We plan to pretreat rats with vehicle (s), propranolol or propranolol plus mifepristone to simulate the conditions of control animals, animals with adrenal demedullation (the lack of epinephrine and thus, the activation of BAR receptors) and animals with total adrenalectomy (the lack of both stress hormones, epinephrine and corticosterone, and thus, the BAR and GR receptor activation), respectively.

2) In the second experiment we will examine the effect of antagonizing GR receptor alone in modulation of systemic and pulmonary effects of ozone in rats. We will pretreat rats with vehicle or mifepristone to antagonize GR receptors and then examine how ozone-induced systemic and pulmonary effects are diminished by inhibiting GR receptors. This antagonist alone is not included in the first experiment since the vehicle control and treatment protocol is different and adding this group alone will make the experiment technically large to handle. Moreover,

the effectiveness of this receptor antagonism will be evident in the first experiment and the results from the first experiment will inform the need for doing the second experiment.

3) Since we have observed that ozone induced lung injury and inflammation are associated with increased circulating epinephrine, we will examine if ozone induced injury and inflammation are exacerbated in rats pretreated with BAR agonist, Clenbuterol (mimicking epinephrine action).

These three experiments will allow us to gather evidence for the role of stress hormones and their receptors in mediating ozone-induced systemic and pulmonary effects. Since this stress response is not specific to only ozone, these results will have wider implications for many environmental stressors and will provide new information on how stressors, in general, can modulate health effects. BAR and GR are present virtually in all organs in the body, and knowing their role in ozone-induced systemic and pulmonary effects will allow us to establish a potential cause for how air pollutants that are inhaled in the lung can produce or exacerbate chronic diseases, such as cardiovascular, metabolic, and neuronal. This research will have implication for risk assessment in general, and will also shed light on the mechanism of susceptibility variations involving stress response pathway impairment. In essence, understanding how ozone can induce systemic effects is important for regulatory standard setting, developing mitigation strategies, and providing a link between environmental factors and incidence of chronic diseases.

2. Scientific rationale for proposed animal use.

a. Why is the use of animals necessary?

The use of animals is necessary in order to understand the complex systemic changes in multiple organs that occur after exposure to pollutants in animal models. We have demonstrated that the exposure of healthy rats to pollutants can produce profound systemic response through neuronal stress pathway. In vitro experimental approach will not be appropriate for determination of systemic health effects and assessing neuronal stress effects on multiple organs. Following a bibliographic search in Pubmed, no validated accepted non-animal methods have been identified to properly mimic inhalation exposures and the subsequent assessment of complex systemic metabolic response.

b. Justify the species requested:

National Institute of Health guidelines recommends the use of rats to study human cardiovascular and metabolic diseases. Rat has been also a preferred animal model for the study of cardiovascular injury from air pollution. Moreover metabolic disorders are better modeled in rats than in mice or in lower vertebrates. Due to the longstanding use of rats for toxicological, cardiovascular, and metabolic studies the necessary databases, reagents, and species-specific assays have been developed, verified to be accurate, and are commercially available. Toxicology data are available for rats to correlate findings of air pollution health effects. We have done a number of toxicological studies using Wistar Kyoto (WKY) rats. Since the studies anticipated under this LAPR involve continuation of our previous studies using WKY rats for examining cardiovascular and metabolic health effects, we propose using this strain of rats to understand how stress response mediates systemic and pulmonary effects of ozone.

3. How was it determined that this study is not unnecessary duplication?

Pubmed and literature searches in Google Scholar performed in February 2016 failed to identify any published studies that investigated neurohormonal stress response in ozone induced systemic metabolic and pulmonary injury inflammation alterations. Using search terms, “ozone” and “stress” and “metabolism” and “beta receptors” yielded three references, one involving brain lipid peroxidation and estradiol in female rats, one involving guinea pig trachea and one involving a study in fish. However, using search terms, “ozone” and “stress” and “metabolism” and “glucocorticoid receptors” yielded no references. We have the current knowledge through direct interaction with scientists in the field and through scientific meetings, and believe that our experiments are not duplicated anywhere else.

SECTION B - In Vivo Procedures

1. Briefly describe the experimental design. Include descriptions of the age, weight and sex of the animals. Supplementary information may be attached at the end of the LAPR, but please include critical information within the body of the LAPR.

The experimental designs for three proposed experiments are explained below.

1) The effect of antagonizing beta adrenergic receptors (BAR) or BAR plus glucocorticoid receptors (GR) on ozone-induced systemic and pulmonary alterations.

Hypothesis: Rats pretreated with BAR or BAR plus GR receptor antagonists will be protected from systemic and pulmonary effects of ozone.

Experimental design: Rats will be treated with vehicle-1 (saline, 1 mL/kg intraperitoneal) or BAR receptor antagonist propranolol (10 mg/kg, in 1mL saline/kg body weight, intraperitoneal); or vehicle-2 (saline, 1mL/kg, intraperitoneal plus pharmaceutical grade corn oil, 1mL/kg, subcutaneous) or Propranolol (10 mg/kg, in 1mL saline/kg body weight, intraperitoneal) plus Mifepristone (30 mg/kg, in 1mL corn oil/kg body weight, subcutaneous) once a day (in the morning between 6 am-7 am) for 7 days prior to the beginning of exposure and until the day of final exposure/necropsy (a total of 8 days). The doses, routes of exposure and times for drugs treatment in rats have been selected based on prior published papers using these receptor antagonists (some papers are attached for reference).

After 7 days of vehicle or drug treatments, rats will be exposed to air or 0.8 ppm ozone for 4hrs for either 1 day or 2 consecutive days. We will analyze the effects of ozone at 1 day and 2 day post-exposure. This protocol is used in several of our prior studies because, 1) 1 day time point allows us to capture 1 day effect (1 day group, without prior animal manipulation such as GTT) where although inflammation and injury are not maximum, gene expression changes are readily apparent, and systemic changes are also clear, 2) 2-day group allows us for the performance of GTT after 1 day exposure, 3) and examine 2-day effect where pulmonary injury and inflammation are readily apparent, while the second day ozone exposure reinitiates most of the acute effects observed on first day. We will perform necropsy immediately following euthanasia, collect blood samples for hormonal and metabolites as well as inflammation analysis. We will collect lung tissue to examine injury and inflammation via bronchoalveolar lavage. We will collect spleen, liver, muscle and tibia marrow for assessment of inflammatory immune cell types. Brain tissues will be collected and frozen or fixed for examining neuronal effects of ozone and BAR and GR receptor antagonism.

This experiment will include a total of 128 rats (n=8 x 4 treatments x 2 exposures x 2 time points) plus 20 cage control rats (4 for each drug treatment x 4 treatments and 4 without any treatment).

2) Experiment #2: The effect of glucocorticoid receptor antagonism in ozone-induced systemic and pulmonary effects.

Hypothesis: Rats pretreated with GR receptor antagonist, mifepristone will be protected from systemic and pulmonary effects of ozone.

Experimental design: Rats will be treated with vehicle (pharmaceutical grade corn oil, 1mL/kg, subcutaneous) or Mifepristone (30 mg/kg, in 1mL corn oil/kg body weight, subcutaneous) once a day (in the morning between 6 am-7 am) for 7 days prior to the beginning of exposure and until the day of final exposure/necropsy. The dose, routes of exposure and times for drug treatment in rats have been selected based on prior published papers using this receptor antagonists (some papers are attached for reference).

After 7 days of vehicle or drug treatment, rats will be exposed to air or 0.8 ppm ozone for 4hrs/day for either 1 day or 2 consecutive days. We will analyze the effects of ozone immediately after 1 day and immediately after 2 day exposure. Exposure and necropsy will be performed as described in Experiment 1.

This experiment will include a total of 64 rats (n=8 x 2 treatments x 2 exposures x 2 time points).

3) Experiment #3: The effect of beta adrenergic receptor agonist on ozone-induced systemic and pulmonary alterations.

Hypothesis: Rats pretreated with BAR receptor agonist will be more susceptible to ozone induced systemic and pulmonary effects.

Experimental design: Rats will be treated with vehicle (saline, 1 mL/kg subcutaneous) or BAR receptor agonist Clenbuterol (0.5 mg/kg, in 1mL saline/kg body weight, subcutaneous) once a day (in the morning between 6 am-7 am) for 7 days prior to the beginning of exposure and until the day of final exposure/necropsy. The dose, routes of exposure and times for drug treatment in rats have been selected based on prior published papers using this receptor agonist (some papers are attached for reference).

After 7 days of treatment rats will be exposed to air or 0.8 ppm ozone for 4hrs for either 1 day or 2 consecutive days. We will analyze the effects of ozone at 1 day and 2 day post-exposure. Exposure and necropsy will be performed as described in Experiment 1.

This experiment will include a total of 64 rats (n=8 x 2 treatments x 2 exposures x 2 time points).

All ozone-exposed animals are classified as Category E due to unrelieved pain/distress, while all other animals are Category C.

2. Justify the number of animals. Include explanation (e.g., biological, statistical, regulatory rationale) for the number of animals needed for each treatment group, and the overall number requested for the duration of the LAPR.

We have chosen 8 male rats (12-14 week old) per experimental treatment group as we have found that this is the minimal number of animals needed to reduce variance and exhibit statistically significant differences that reflect real biological changes in systemic and metabolic effects.

Experiment #1 will include a total of 128 rats plus 20 cage control rats as below.

-4 Drug treatments: 2 controls plus 2 drug treatments
-2 Exposures: air or ozone 0.8 ppm
-2 Time points: 4hr/day, 1 day exposure or 4hr/day, two consecutive days of exposure
-8 rats/group for appropriate statistical power and accounting for within group variability

Of 20 cage control rats,
-4 will have no drug treatment,
-4 will have saline vehicle
-4 will have propranolol
-4 will have saline + corn oil vehicles
-4 rats will be treated with propranolol plus mifepristone

These control rats will allow us to separate cage effects due to housing rats in wire mesh cages for exposure.

Experiment #2 will include a total of 64 rats-

-2 Drug treatments: 1 vehicle control plus 1 drug treatment
-2 Exposures: air or ozone 0.8 ppm
-2 Time points: 4hr/day, 1 day exposure or 4hr/day, two consecutive days of exposure
-8 rats/group for appropriate statistical power and accounting for within group variability

Experiment #3 will include a total of 64 rats-

-2 Drug treatments: 1 vehicle control plus 1 drug treatment
-2 Exposures: air or ozone 0.8 ppm
-2 Time points: 4hr/day, 1 day exposure or 4hr/day, two consecutive days of exposure
-8 rats/group for appropriate statistical power and accounting for within group variability

All three experiment will include 276 rats in total.
All ozone-exposed animals are classified as Category E due to unrelieved pain/distress (128), while all other

animals are Category C (128).

3. State how many animals over the study period are expected to be used under the following three categories of pain/distress (USDA nomenclature as defined in the instructions): Please enter numbers only.

Categories	Adults	Offspring
C) Minimal, transient, or no pain/distress:	148	
D) Potential pain/distress relieved by appropriate measures:		
E) Unrelieved pain/distress:	128	

4. Does this LAPR include any of the following:

☐ Restraint (>15 Minutes) ☐ Survival surgery

☒ Food and/or water restriction (>6 Hours) ☐ Non-survival surgery

a. Please provide a scientific justification. Describe how animals will be monitored, how health status will be tracked, and what records will be maintained.

The performance of glucose tolerance test and the assessment of metabolic markers requires prior 6-8 hours fasting in rodent studies to obtain stabilized baseline values for metabolites which are highly influenced by food intake. Glucose tolerance testing will be done immediately after ozone exposure in 2 day exposure group for which fasting glucose measurements are needed. Animals will be fasted during 4 hr ozone exposure and then during ~2.5 hours glucose tolerance testing. Animals will be monitored hourly during ozone exposure for signs of discomfort and then continuously during glucose tolerance testing during which a large bolus of glucose in injected, which might eliminate some effect of food restriction.

5. Category C procedures. Describe each procedure separately, include details on the following:

a. Treatments (e.g., dosages, duration of exposure, route, volume, frequency):

1) Saline: Pharmaceutical grade sterile saline will be injected intraperitoneally (1 mL/kg) in vehicle control rats daily during the experimental duration.

2) Propranolol: Propranolol has been widely used clinically and in numerous experimental studies. We will administer propranolol daily intraperitoneally at a 10 mg/kg dose level in 1mL/kg pharmaceutical grade saline in rats.

3) Mifepristone: This GR and progesterone receptor antagonist is also widely employed clinically and in many experimental studies. Mifepristone will be dissolved in corn oil at 30 mg/mL concentration, prepared fresh daily and administered subcutaneously at 1mL/kg (30 mg/kg) dose level daily during proposed experimental duration.

4) Clenbuterol: This BAR receptor agonist is widely employed in experimental studies. Clenbuterol will be dissolved in saline at 0.5 mg/mL and administered subcutaneously at 1mL/kg dose once daily for during proposed experimental duration.

5) Corn oil (https://www.spectrumchemical.com/OA_HTML/chemical-products_Corn-Oil-NF_CO136.jsp?section=26110): Pharmaceutical grade 1mL/kg, subcutaneous daily for the duration of the study.

6) Glucose: During GTT, after the baseline blood glucose measurement, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 grams/kg body weight/10mL pharmaceutical grade saline). The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) from Sigma Aldrich and dilute to 20% using pharmaceutical grade saline. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection. In our prior studies animals showed no signs of peritonitis or infections in the abdomen after repeated GTT over a three month period. Their weight gains were not affected by GTT. The scientific staff involved in the study will watch for signs of abdominal pain such as lateral or vertical stretching, and weight loss daily until the necropsy.

No deleterious effects of these drug treatments are expected in animals. All drug treatments will occur for a maximum of 8 days, once per day. After the first treatment rats will be monitored for 2-3 hours for possible discomfort. Then during each subsequent treatment in the morning prior to exposure rats will be monitored for possible discomfort and signs of weight loss. Attending veterinarian will be notified for advice if significant weight loss is observed.

b. Survival Blood Collections (method, volume, frequency):

Blood collection for measurement of insulin will be performed immediately following day 1 of air or ozone exposure (coordinated with GTT for only 2-day exposure group). The time line specified in the following section (B5c). Two blood draws will be done on each rat; one at baseline and one 30 minutes post glucose injection. Rats will be restrained for ~3-5 minutes in a conical nose-only restrainer that we use for inhalation studies. These tubes will be anchored by straps at the edge of lab platform using an anchoring device custom made for this purpose. The rat tails will be warmed and cleaned with warm wet cloth. The tail will be wiped cleaned and lateral vein will be visualized. Winged infusion needles (21-25G) will be used to insert in the tail vein. Blood will be allowed to drip in Microtainer tube with a serum separator plug (~200 microliter). The hemostasis will be achieved by pressure and clean sterile gauze. Thirty minutes after injection of pharmaceutical grade glucose for GTT (2g/kg/10mL; intraperitoneal) the second blood collection on same rat will be done as shown above. The hemostasis will be achieved as above by pressure and clean sterile gauze. **Exemption 6** and I have performed this technique, under LAPR 16-03-003, amendment #12. Since blood collection will occur two times on same animal, we will alternate between right and left tail vein (~3 inch from the tip of the tail).

GTT protocol and glucose intraperitoneal injection: Rats will be fasted during exposure periods prior to GTT (a total of 6-8 hours fasting). For each test, the baseline blood glucose levels will be measured following ~6 hours fasting. Since this food restriction technique is commonly used, we do not expect any deleterious effects. Blood will be collected by pricking the tip of the tail with a 25 gauge sterile needle following wiping with an alcohol swab and clean dry gauze. About 1 microliter blood droplet will be brought into contact with the glucometer strip (attached to Bayer Contour Glucometer) (0.6 micro liter of blood is aspirated in the strip). Glucose is measured within three seconds and recorded. Once baseline glucose is measured, pharmaceutical grade glucose will then be injected intraperitoneally (2 grams/kg body weight/10mL) and blood glucose will be measured at 30 min interval for four times in each rat. For GTT, each rat will have a total of 5 glucose measurements (baseline plus four times following intraperitoneal injections of glucose). The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) from Sigma Aldrich. Glucose solution will be made fresh each time using new pharmaceutical grade saline vials. Sterile syringe and needles will be used for each rat for intraperitoneal injection. All GTT testing will be done in room A552. During GTT tail vein blood collection will be done as indicated above.

Timeline for GTT: GTT is performed right after exposure (~11am start time) as stated above:

-Blood glucose measured by tail prick at 0 min and tail vein blood collected as above.
-Glucose injected intraperitoneally right after the zero minute blood glucose testing
-Glucose measured by tail prick at 30 min and and tail vein blood collected as above
-Glucose measured by tail prick at 60 min
-Glucose measured by tail prick at 90 min
-Glucose measured by tail prick at 120 min

c. Testing methods (including non-stressful dietary restrictions/modifications, mild non-damaging electric shock):

Respiratory monitoring using whole body Plethysmography - EMKA system: Whole body plethysmography measurements, will be performed only on rats assigned 2-day group for all 3 experiments. Whole body plethysmography will be performed prior to the start of drug treatment, after 6 days of drug treatment, and immediately following first and second day of air or ozone exposure. All plethysmography measurements will be done at 10-11 am in the morning to reduce diurnal variation. Breathing parameters will be measured using EMKA system. Breathing parameters are monitored in freely moving rats. No restrain is used. Rats are placed in plethysmography chambers while pressure parameters are collected to compute breathing frequency, minute volume, respiratory time and enhanced pause before and after exposures. The rats are placed in a whole-body plethysmography for 5-10 min. We have routinely used this duration for acquisition of breathing parameters which has been adequate for stabilization and recording. No restraint or other stresses are involved in this process. This measurement allows in depth evaluation of lung health in unrestrained freely moving rats.

d. Animal restraint and confinement beyond routine housing and handling. Include a description of the type of restraint device, acclimation to device, duration of restraint:

Nose-only tube restraint for tail vein blood collection: Rats will be acclimatized to conical plastic nose-only inhalation tubes for 5 minutes each day (designed for nose-only inhalation purpose) for 2 days prior to collection of blood. Animals will be placed in the nose-only restraining tubes on the holding rack during acclimation period. Then rats will be restrained for a period of 5 minutes to nose-only tubes during blood collection by tail vein as planned with GTT.

e. Breeding for experimental purposes (e.g. length of pairing, number of generations):

none

f. Describe how animals will be identified and monitored. Include description of identification procedures. (For example, if transponders are used, how are the animals prepared?) Include frequency of observations and by whom:

For air exposure of 4hr/day for 1 or 2 consecutive days, each animal will be identified by a unique identification number marked using permanent ink pen on tail and all cages will be labelled with details of treatments and exposure conditions. After the first treatment rats will be monitored for 2-3 hours for possible discomfort. Then during each subsequent treatment in the morning prior to exposure rats will be monitored for possible discomfort and signs of weight loss. During exposure, Exemption 6Exemption 6Exemption 6Exemption 6 Exemption 6 will monitor animals, at least once per hour for entire exposure duration. During post exposure period of up to 20 hours, rats will be monitored by Exemption 6Exemption 6Exemption 6 in the evening and then in the morning for visible signs of discomfort and weight loss. All animals will be monitored for signs of illness (huddling, isolation with ruffled coat, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No weekend exposures are scheduled, and the animals will be necropsied within 48 hours of the start of the first ozone exposure. No significant weight loss due to ozone is expected in any of the experimental conditions.

6. Non-surgical Category D or E procedures. Describe each procedure separately, include details on the following (Also fill in Section B.9).

a. Treatments (e.g. dosages, duration of exposure, route, volume, frequency):

For ozone-exposed animals (Category E), rats will be exposed to 0.8 ppm ozone 4hrs per day for either 1 or 2 consecutive days. Ozone at this concentration in Wistar Kyoto rat produces lung inflammation, hypothermia, and a stress response which resolves on its own after one day upon discontinuation of exposure.

During air or ozone inhalation exposures of maximum of 4hrs/day (whole-body), rats are placed in individual stainless steel wire mesh cages, and food and water are withheld while the rats are being exposed. Ozone exposures will be done using whole body exposure system in large Hazelton-type inhalation chambers where each rat is placed in wire-mesh cages. The rats are weighed daily following each exposure and examined for any visible clinical signs of discomfort or poor health. The rats are also checked after each exposure when they are returned to home cages. All findings are recorded. We have done a number of ozone inhalation studies in the previous LAPR (#16-03-003).

b. Blood Collection (Provide a description of the procedure including method, volume, and frequency if appropriate. Indicate if the procedure is survival or terminal. Include preparatory methods, descriptions of incisions, etc.):

For ozone-exposed (Category E) animals, animals will be have food and water restriction during the ozone exposure. Blood collection will be performed during GTT for insulin measurement in these animals. At base line and immediately following 30 minutes after bolus glucose injection, ~200 microliter (each time) blood samples will be collected through tail vein in a serum collection tube. We have noted that ozone exposure abolishes insulin release into the circulation in response to glucose injection. It has been shown that epinephrine plays a role in blunting the insulin secretion. Since we are examining the role of beta receptor antagonist and agonist, we believe that it is important to determine if this metabolic effect of ozone is impacted by B2 and GR receptor antagonists.

Blood collection for measurement of insulin secretion will be performed immediately following day 1 of air or ozone exposure (coordinated with GTT for only 2d-ay exposure group). Two blood draws will be done on

each rat; one at base line and one 30 minutes post glucose injection. Rats will be restrained for ~3-5 minutes in a conical nose-only restrainer that we use for inhalation studies. These tubes will be anchored by straps at the edge of lab platform using an anchoring device custom made for this purpose. The rat tails will be warmed and cleaned with warm wet cloth. The tail will be wiped cleaned and lateral vein will be visualized. Winged infusion needles (21-25G) will be used to insert in the tail vein. Blood will be allowed to drip in Microtainer tube with a serum separator plug (~200 microliter during each draw). The hemostasis will be achieved by pressure and clean sterile gauze. Thirty minutes after injection of pharmaceutical grade glucose for GTT (2g/kg/10mL; intraperitoneal) the second blood collection on same rat will be done as shown above. The hemostasis will be achieved as above by pressure and clean sterile gauze. Then rats will be relocated in their respective cages. Exemption 6 and I have performed this technique, under LAPR 16-03-003, amendment #12. Since blood collection will occur two times on same animal, we will alternate between right and left tail vein (~3 inch from the tip of the tail).

c. Testing methods:

none

d. Restrictions placed on the animals' basic needs (e.g., food and/or water restriction, light cycles, temperature). Provide details regarding the length of restriction. Describe the method(s) for assessing the health and well-being of the animals during restriction. (Amount of food or fluid earned during testing and amount freely given must be recorded and assessed to assure proper nutrition.):

For ozone-exposed (Category E) animals, animals will have food and water restriction during the ozone exposure 4hrs each day for two consecutive days. For animals assigned 2 days of exposure, immediately after their first day after ozone exposure, GTT will be performed which lasts for about 2.5 hours. Thus for these animals food restriction will last for 6-8 hrs maximum that includes ozone exposure and GTT times.

e. Describe how animals will be monitored (e.g., frequency of observations, by whom):

Rats will be monitored hourly during 4 hrs whole body ozone exposure in the inhalation chambers for visible signs of discomfort by Exemption 6Exemption 6Exemption 6 . After completion of exposure, all animals will be monitored for signs of illness (huddling, isolation with ruffled coat, shivering, development of hindered movement, etc) and if any adverse effect is observed, we will consult with the staff veterinarian and follow the recommended protocol. Visual inspection of labored breathing and isolation will be carefully monitored. No weekend exposures are scheduled, and the animals will be necropsied within 48 hours of the start of the ozone exposure. No significant ozone-related weight loss is expected during exposure.

f. Analgesia (Category D Procedures) - list drugs, dosages, route of administration and frequency:

none

g. If treatment-related deaths are expected, this must be thoroughly justified. Death as an endpoint is highly discouraged:

No ozone-related deaths are expected.

7. Surgical Category D and E procedures. Indicate if the surgery is survival or terminal. Describe each surgical procedure separately, include details on the following (Also fill in Section B.9)

a. Complete description of surgical procedure including presurgical preparation, aseptic technique, surgical closure, etc:

none

b. Anesthetic regimen (Drugs, dosages, volume, route of administration and delivery schedule). The use of paralytic or neuromuscular blocking agents w/o anesthesia is prohibited:

n/a

c. Postoperative care (thermal support, special feeding, responsible personnel, removal of sutures/staples, frequency and duration of monitoring including weekend and holiday care):

n/a

d. Post operative analgesics (drugs, dosage, and volume and route of administration, frequency):

n/a

e. Will any animal be subject to more than one surgical procedure over the course of its lifetime, either here at NHEERL or elsewhere?

○ Yes ● No

f. Identify any surgical procedures performed at other institutions or by vendors:

n/a

8. Humane interventions (for treatments/procedures in all categories).

a. What resultant effects, if any, do the investigators expect to see following procedures or treatment? Please include transitory as well as permanent effects. Examples might include lethargy, ataxia, salivation or tremors. Indicate the expected duration of these effects.

No adverse effects are expected from any of the drug treatments. Ozone at 0.8ppm concentration for 4hrs in Wistar Kyoto rat produces lung inflammation, hypothermia, and a stress response which resolves on its own after one day upon discontinuation of exposure. Ozone is expected to induce labored breathing. In the event of expected or unexpected deleterious effects, the Attending Veterinarian will be immediately notified for guidance on subsequent steps including euthanasia. Animals will be isolated in a clean control atmosphere and observed for recovery trends, and may be transferred to the training colony if recovered. No deleterious effects of these non-surgical procedures, however, are expected.

b. State the criteria for determining temporary or permanent removal of animals from the study. Describe actions to be taken in the event of deleterious effects from procedures or chemical exposures. Describe actions to be taken in the event of clinical health problems not caused by procedures or exposures.

Any animals displaying signs of illness (weight loss of >10% occurs overnight, huddling, isolation with ruffled coat, shivering, development of hindered movement, labored breathing and isolation etc) 20 hours following the first exposure will be considered for permanent removal as per advice of the attending veterinarian. Ozone induced labored breathing is reduced the following day to ~20% of the value obtained immediately following 4 hour exposure in this rat model. If animals exhibit signs of labored breathing at 20 hours after their first exposure (prior to second day exposure), Attending Veterinarian will be consulted. If animals displayed signs of labored breathing after 24 hours post first exposure, animal will be euthanized."

9. Alternatives to pain and distress (Category D and E Procedures only). Provide narrative regarding the sources consulted to ascertain whether acceptable alternatives exist for potentially painful/distressful procedures. Include databases searched or other sources consulted, the date of the search and years covered by the search, and key words and/or search strategy used. Assistance with searches is available through the EPA Library Staff.

Although searching PubMed using search terms, "ozone toxicity and alternative to pain" provided no reports, "ozone toxicity and therapeutic" provided 346 references which included in vitro and in vivo experiments where inhibitors of oxidative stress and inflammatory cell signaling markers were shown to be effective in reducing ozone toxicity in cells and in animals. Using any therapeutic intervention reported in the literature to reduce toxicity, other than what is proposed in this study, will be counterintuitive to our hypothesis and thus will be inappropriate.

SECTION C - Animal requirements

Describe the following animal requirements :

1. Indicate the number of animals required over the study period for this protocol. Please enter numbers only.

a. Animals to be purchased from a Vendor for this study:	276
b. Animals to be transferred from another LAPR: LAPR Number that is the source of this transfer:	
c. Animals to be transferred from another source:	
d. Offspring produced onsite (used for data collection and/or weaned):	
e. TOTAL NUMBER of animals for duration of the LAPR	276

2. Species (limited to one per LAPR): Rat(s)

3. Strain: WKY rat(s)

Describe special requirements for animals with altered physiological responses (e.g., genetically altered, aged)

None.

4. Sources of animals:

Charles River Laboratories, Inc.

5. Provide room numbers where various procedures will be performed on animals:

1. Rats will be housed in one of the animal housing rooms upon arrival Exemption 6 Exemption 6 or other available room) and during non-exposure periods.
2. During exposure, rats will be transferred in an original rack with rats housed in home cages to green floor inhalation exposure rooms (whole body exposures – Exemption 6 Exemption 6 Exemption 6. Once the exposures are complete rats will be transferred to their home cages in the same rack and moved back to the animal holding room.
3. Whole body plethysmography, glucose tolerance testing and tail vein blood collection will be done in room Exemption 6 Exemption 6 or other available room.
4. The day of necropsy after exposure, animals will be transferred to Exemption 6 Exemption 6 for necropsy using transfer cages with beta chips bedding and filtered cage tops.

6. Will any animals be housed in areas other than the animal facility longer than 12 hours? If so, state location. Such areas require prior IACUC approval as a satellite facility before LAPR can be reviewed.

No *Room Numbers:* n/a

7. Describe any transportation and containment methods involved in moving animals between EPA buildings, or between EPA and other institutions (excluding any commercial shipments)

None.

8. Describe any unusual housing or husbandry requirements, or acclimation requirements. Justify any treatment beginning less than 3 days after arrival.

None.

9. Describe special assistance requested of the animal contract staff, including procedures and dosing. NOTE, this request must be submitted separately to the Animal Resources Program Office (ARPO)

None.

10. Housing and Enrichment.

The IACUC encourages the use of environmental enrichment whenever possible (see IACUC website for details). Provide details on how the animals will be housed, including type of cage (e.g., solid bottom or wire screen), bedding material, number of animals per cage, and environmental enrichment. Note that housing rodents individually without environmental enrichment requires justification.

All animals will be pair housed in solid bottom caging with beta chips bedding in building A during non-exposure periods as per the IACUC guidelines. Nesting material (Envirodry) will be placed in cages for enrichment.

SECTION D - Agents Administered to Animals

1. Identify all hazardous and non-hazardous agents to be administered to living animals. For agents requiring a Health and Safety Research Protocol (HSRP), provide the title of the approved HSRP for each such agent. If no protocol is required for an agent deemed potentially hazardous (e.g. nanoparticles, recombinant DNA), describe the safety precautions to be used.

Provide maximum dosing levels and route-appropriate LD50s (where available) for each agent used for dosing.

1) Ozone inhalation exposures: Ozone exposure will occur in whole body exposure chambers to a maximum of 0.8 ppm concentration.

The LC50 for ozone is 4.8 ppm in rats (4800 ppb/ 4 hours/ inhalation/ rat). HSP copy attached (Title: Small Animal Inhalation Exposures to Nitrogen Dioxide and ozone).

2) Saline: Pharmaceutical grade sterile saline will be injected intraperitoneally (as control for propranolol) or subcutaneously (as control for Clenbuterol) (1 mL/kg) in vehicle control rats daily during the experimental duration.

3) Propranolol: Propranolol has been widely used clinically and in numerous experimental studies. We will administer propranolol daily intraperitoneally at a 10 mg/kg dose level in 1mL/kg pharmaceutical grade saline in rats. Acute oral LD50 in mice is 320 mg/kg body weight.

4) Mifepristone: This GR and progesterone receptor antagonist is also widely employed clinically and in many experimental studies. Mifepristone will be dissolved in corn oil at 30 mg/mL concentration and administered subcutaneously at 30 mg/kg/mL dose level daily during proposed experimental duration. Oral rat LD50 value for this chemical is 980 mg/kg.

5) Clenbuterol: This BAR receptor agonist is widely employed in experimental studies. Clenbuterol will be dissolved in saline at 0.5 mg/mL and administered subcutaneously at 1mL/kg dose once daily for during proposed experimental duration. Oral LD50 value for Clenbuterol in rat is 159 mg/kg and intraperitoneal LD50 for rat is 67 mg/kg.

6) Pharmaceutical grade corn oil: (https://www.spectrumchemical.com/OA_HTML/chemical-products_Corn-Oil-NF_CO136.jsp?section=26110): 1mL/kg, subcutaneous daily for the duration of the study. Acute oral LD50 is >100mL/kg for rat but no data are available for dermal or subcutaneous toxicity.

7) Glucose: During GTT, pharmaceutical grade glucose will be injected intraperitoneally (maximum of 2 grams/kg body weight/10mL pharmaceutical grade saline). The 10ml/kg body weight volume has been used in published studies for rats. We will purchase pharmaceutical grade sterile glucose solution (40% concentration) from Sigma Aldrich and dilute to 20% using pharmaceutical grade saline.

Researchers will handle all agents in accordance with good industrial hygiene and safety practices. Lab coat, safety glasses and gloves will be worn when handling these chemicals. Drug preparations will be done in chemical safety hood. All animals will be monitored periodically during exposure.

2. Describe compounds to be administered to animals.

a. Are all substances pharmaceutical grade? If not, provide a scientific justification for the use of non pharmaceutical grade compounds.

Saline, Corn oil and glucose solution are pharmaceutical grade. Propranolol, mifepristone, and Clenbuterol are research grade chemicals. All published studies which we are following (some are attached have used the ultra pure research grade chemicals prepared in appropriate vehicles indicated in these studies. We will used previously published protocols for these drugs in the proposed studies. The use of these research grade drugs is necessary for comparing research results from our studies to those that have been published using these drugs.

b. Describe any plans to administer human or animal tissues, blood or body fluids to the animals in the LAPR. Provide information to assure that such material is pathogen free. Indicate what safety precautions are necessary for handling the material.

No.

c. Provide a statement regarding any safety precautions necessary for handling any of these materials.

n/a

NOTE: Any unresolved health/safety questions which arise during IACUC review of this LAPR will require consultation with the Safety, Health, and Environmental Management Office.

SECTION E - Personnel Training and Experience

1. Identify all project personnel conducting animal experimentation. Specify the techniques for which they have responsibility, and their relevant training and experience. Additional personnel may be added to the table below as a group (by Division) for Category C procedures. By so doing you are giving assurance that these personnel have received all required training and are qualified to perform the Category C techniques requested.

Use this area to type in additional personnel information not available in the table drop-down lists:

Hint: *The names in the first 2 lines of the table below are filled automatically from the Principal Investigator & Alternate Contact fields. A new line will be made available when a name is selected & upon leaving the name field (i.e. tabbing or clicking in another field).*

NAME	ROLE	SPECIFIC RESPONSIBILITY	RELEVANT TRAINING
Exemption 6	Principal Investigator	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6 Exemption 6 Exemption 6 Exemption 6	Technical Staff	Assist in study planning, animal handling, and necropsy.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Student	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Two years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Plan study, prepare protocols, and oversee the experiment. Assist in animal handling, testing, and necropsy.	Four years of experience working with rats; all relevant NHEERL required training completed.
Exemption 6	Technical Staff	Assist in study planning, animal handling, exposure, and whole body plethysmography.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Post-Doc	Assist in animal handling, testing, and necropsy.	Eight years of experience working with rats at various institutions; all relevant NHEERL required training completed.
Exemption 6	Student	Assist in animal handling, GTT testing, and necropsy.	Five years of experience working with rats; all relevant NHEERL required training completed.
Exemption 6,	Associate	Assist in animal	Twenty years of experience working with

Exemption 6 Exemption 6	Principal Investigator	handling and brain tissue collection	rats at EPA and other institutions; all relevant NHEERL required training completed.
Exemption 6	Technical Staff	Assist in animal handling, GTT testing, and necropsy.	Twenty years of experience working with rats at EPA and other institutions; all relevant NHEERL required training completed.
RTP-NHEERL	Tech Support	Category C Procedures	All NHEERL required training is complete.

SECTION F - Animal Breeding Colonies

This section pertains to the breeding of animals for maintenance of ongoing animal colonies. Do not include breeding that is part of experimentation and accountable under Section C.

Describe:

1. Estimated number of breeding pairs and liveborn per year n/a

2. Breeding protocols and recordkeeping n/a

3. Methods for monitoring genetic stability n/a

4. Disposition of all offspring and retired breeders that are not used in accordance with the procedures described in this LAPR n/a

SECTION G - Euthanasia

1. When will the animals be euthanized relative to experimental procedures?

Animals will necropsied for blood sample and tissue collections following terminal euthanasia, after 1 or 2 days exposure

2. Describe the euthanasia techniques:

Method(s): Euthanasia plus exsanguination

Agent(s): Pentobarbital injectable preparations, diluted with sterile saline to achieve maximum of 200 mg/ml concentration

Dose (mg/kg): 150-250 mg pentobarbital/kg

Volume: 0.75 – 3.0 ml as needed

Route: Intraperitoneal

Source(s) of information used to select the above agents/methods:

Veterinary Staff
IACUC

3. Provide justification and references for any euthanasia agent or method that is not consistent with recommendations of the American Veterinary Medical Association (AVMA) Guidelines for Euthanasia (e.g., cervical dislocation or decapitation without anesthesia; cervical dislocation in rodents weighing more than 200 grams).

None.

4. Describe how death is to be confirmed.

Vital organ section

SECTION H - Disposition of Used and Unused Animals

Describe the disposition of any animals remaining after project completion.

Transferred to another study

The IACUC encourages investigators to reduce the overall number of animals used at NHEERL. Would you consider transferring any unused animals from this LAPR to another approved LAPR?

● Yes ○ No

SECTION I - Assurances

1. Animals will not be used in any manner beyond that described in this application without first obtaining formal approval of the IACUC.
2. All individuals involved in this project have access to this application, are aware of all EPA policies on animal care and use, and are appropriately trained and qualified to perform the techniques described.
3. Thorough consideration of the three "R"'s (Replacement, Reduction, Refinement) has been given, as applicable, to a. the use of animals, and b. procedures causing pain or distress (with or without analgesia/anesthesia), including death as an endpoint. The minimum number of animals required to obtain valid experimental results will be used.
4. The Attending Veterinarian has been consulted in regard to any planned experimentation involving pain or distress to animals.
5. The IACUC and Attending Veterinarian will be promptly notified of any unexpected study results that impact the animals' well-being, including morbidity, mortality and any occurrences of clinical symptoms which may cause pain or indicate distress.
6. All procedures involving hazardous agents will be conducted in accordance with practices approved by the Safety, Health, and Environmental Management Office.
7. I certify that I am familiar with and will comply with all pertinent institutional, state and federal rules and policies.
8. The IACUC has oversight responsibilities for animal care and use, and may request consultation or feedback regarding the conduct of in vivo procedures, progress and accomplishments, and any problems encountered.

EPA Principal Investigator	Certification Signature Date
Exemption 6 Exemption 6	02/02/2016

Submitted: 02/02/2016

Certification:

Certification by EPA Supervisor (Branch Chief or Division Director) that the project described herein has been reviewed and approved on the basis of scientific merit:

Branch Chief/Division Director	***Approval Date***	***Phone Number***	***Division***	***Mail Drop***
Exemption 6	02/03/2016	Exemption 6	EPHD	MD
		Lotus Notes Address	***Branch***	***Submitted to Branch Chief for Approval***
	by Exemption 6 Exemption 6 /RTP/USEPA/US	Exemption 6 Exemption 6 /RTP/USEPA/US	CIB	02/02/2016 06:02 PM

ATTACHMENTS

19-02-002 PI resp.pdf Propranolol rat ip 15 mg per kg for several days.pdf propranolol 10 mg per kg ip rats.pdf


mifepristone peanut oil 40 mg per kg subcute 4 days.pdf

mifepristone peanut oil 30 mg per kg subcute 1 day.pdf

Corn oil pharmaceutial grade.pdf

Clenbuterol rat subcute 1mg per kg 15 days.pdf

HSRP_form NO2 and ozone exposures 131112 final._778.pdf

Actions

First Update notification sent: 01/04/2017
Second Update notification sent: 01/31/2017
First 2nd Annual notification sent: 01/08/2018
Second 2nd Annual notification sent: 02/02/2018
1st Expiration notification sent:
2nd Expiration notification sent:

History Log: